Table 4 Patient characteristics classified by IL28B SNPs at the time of diagnosis

	TT ($N = 162$)	TG/GG ($N = 66$)	p value
Sex (male/female)	81/81	32/34	0.951
Age (years)	63.3 ± 10.7	63.8 ± 11.9	0.735
Platelets ($10^{-4}/mm^3$)	13.8 ± 5.8	14.3 ± 6.6	0.632
Albumin (g/dL)	4.2 ± 3.0	4.0 ± 0.5	0.528
γGTP (IU/L)	41 ± 39	55 ± 49	0.020
T.Chol (mg/dl)	162 ± 31	157 ± 31	0.237
HCV RNA concentration (kIU/ml)	7,576 ± 10,292	5,069 ± 6,701	0.085
Alpha-fetoprotein (ng/ml)	39.0 ± 152.2	27.3 ± 48.3	0.555
AST (IU/L)	49.6 ± 29.6	51.9 ± 30.5	0.607
ALT (IU/lL)	54.5 ± 53.9	51.6 ± 37.7	0.689
Core a.a. 70R/(Q/H)	106/56	17/49	<0.001
Core a.a. 91L/(M/C)	108/54	38/28	0.253
ISDR	1.2 ± 2.0	0.9 ± 1.5	0.164
IRRDR	5.1 ± 2.4	4.7 ± 2.2	0.207
HCC −/+	115/47	45/21	0.788
IFN −/+	95/67	34/32	0.402

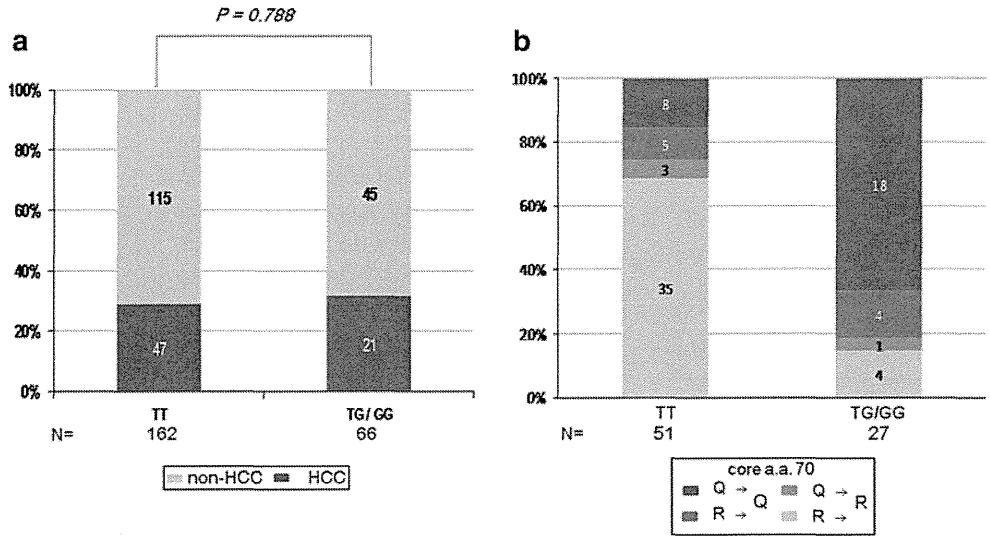

Fig. 4 **a** Association between the state of liver disease and IL28B SNP. **b** Time-dependent core a.a. 70 changes and its relation to IL28B SNP was investigated in 78 patients

of R/(Q/H) at core a.a. 70 was significantly higher in those with the TT alleles than in those with TG/GG ($p < 0.001$). In association of IL28B SNP with HCC development, there was no evident relationship as demonstrated in Fig. 4.

IL28B SNP and time-dependent core a.a. 70 changes

In Fig. 4b, it is demonstrated that the direction of time-dependent core a.a. 70 change was influenced by IL28B SNPs. In IL28B TG/GG patients, 4 (50%) out of 8 patients with the initial core a.a. 70R changed into 70Q, while only 1 (5%) out of 19 patients with the initial core a.a. 70Q changed into 70R, demonstrating that core a.a. 70 tended to change into Q over time in IL28B TG/GG patients ($p = 0.034$). On the other hand, there was no evident changing direction in IL28B TT patients; 5 (13%) out of 40 patients with the initial core a.a. 70R changed into 70Q, while 3 (27%) out of 11 patients with the initial core a.a. 70Q changed into 70R ($p = 0.45$).

Multivariate analysis for independent factors influencing core a.a. 70

To investigate further the relationship between core a.a. 70, the IL28B SNP, and HCC development, we divided the patients according to the specification of core a.a. 70 and

Table 5 Factors related to polymorphism of core a.a. 70

Variables	Univariate analysis ($N = 228$)		Multivariate analysis ($N = 228$)	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Sex				
Female	1	0.415	1	0.812
Male	1.23 (0.74–2.01)		1.08 (0.58–1.99)	
Age (years)				
<65	1	0.216	1	0.855
≥65	1.39 (0.82–2.35)		1.06 (0.57–1.96)	
Platelets (10^{-4}/mm^3)				
>12	1	0.004	1	0.844
≤12	1.76 (1.03–2.99)		1.07 (0.53–2.16)	
Albumin (g/dL)				
>4	1	0.002	1	0.300
≤4	2.28 (1.33–3.91)		1.46 (0.71–3.00)	
γGTP (IU/L)				
<41	1	0.003	1	0.299
≥41	2.32 (1.32–4.09)		1.42 (0.73–2.79)	
ALT (IU/L)				
<41	1	0.040	1	0.573
≥41	1.74 (1.03–2.94)		1.22 (0.62–2.39)	
IL28B				
TT	1	<0.001	1	<0.001
TG or GG	5.46 (2.88–10.30)		5.74 (2.91–11.31)	
HCC				
−	1	<0.001	1	0.046
+	2.98 (1.65–5.37)		2.21 (1.01–4.83)	
Previous IFN therapy				
−	1	0.874	1	0.644
+	0.96 (0.57–1.62)		0.87 (0.47–1.59)	

those factors, as well as clinical factors, were compared in univariate and multivariate analyses. In Table 5, it may be seen that platelets, albumin, γGTP, ALT, the IL28B SNP, and number of patients with HCC development differed significantly between the two groups in univariate analysis. In contrast, successive multivariate analysis demonstrated that the number of patients with HCC development ($p = 0.046$) and the IL28B SNP ($p < 0.001$) were extracted as independent variables correlated with the core a.a. 70 residue (Table 5).

Multivariate analysis for independent factors influencing HCC development

To disclose factors influencing HCC development, multivariate analysis was performed. As shown in Table 6, age, albumin, and core a.a. 70 residue were extracted as independent factors. On the other hand, IL28B SNP was not extracted as one of those factors.

Discussion

In this study, we have documented several important findings. Through the investigation of HCV sequences, including complete HCV ORFs analysis, we have shown that the core a.a. 70 residue and its changes over time are associated with the disease progression as well as HCC development in genotype-1b HCV infection. Specifically, core a.a. 70Q/H was associated with HCC development and disease progression; core a.a. 70 often changed with time and R70Q substitutions were associated with progressive disease, while Q70R substitutions were associated with the stable disease. Moreover, we have shown that the IL28B SNP and core a.a. 70 showed significant linkage. In contrast, we have also shown that HCC development and disease progression were not apparently correlated with the IL28B SNP.

Recently, core amino acids have been reported in several studies to be associated with HCC [12, 21–25]. In

Table 6 Factors related to influencing HCC development

Variables	Univariate analysis ($N = 228$)		Multivariate analysis ($N = 228$)	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Sex				
Female	1	0.161	1	0.190
Male	1.50 (0.85–2.67)		1.69 (0.77–3.71)	
Age (years)				
<65	1	0.006	1	0.004
≥65	2.30 (1.28–4.16)		3.26 (1.46–7.25)	
Platelets (10^{-4}/mm^3)				
>12	1	<0.001	1	0.021
≤12	5.82 (3.11–10.88)		2.59 (1.16–5.82)	
Albumin (g/dL)				
>4	1	<0.001	1	<0.001
≤4	13.75 (6.69–28.24)		7.73 (3.53–16.94)	
γGTP (IU/L)				
<41	1	<0.001	1	0.122
≥41	3.09 (1.70–5.62)		1.87 (0.85–4.13)	
ALT (IU/L)				
<41	1	<0.001	1	0.109
≥41	3.88 (2.06–7.31)		1.98 (0.86–4.56)	
IL28B				
TT	1	0.626	1	0.290
TG or GG	1.17 (0.13–2.17)		0.63 (0.27–1.49)	
Core a.a. 70				
R	1	<0.001	1	0.029
Q/H	2.91 (1.61–5.26)		2.44 (1.09–5.44)	
Previous IFN therapy				
−	1	0.949	1	0.331
+	0.98 (0.55–1.74)		1.46 (0.68–3.16)	

these studies, patients with core a.a. 70Q/H frequently developed HCC with exacerbation of liver damage. In this analysis, we confirmed the previous findings. However, because this association might be a reflection of the core-dependent IFN sensitivity differences often reported in recent studies [12, 22, 25], we restricted the analysis to patients, who were unable to clear HCV RNA previously through IFN-based therapy. Moreover, we also confirmed the relationship of the core sequences and disease development among the populations without a previous history of IFN therapy (data not shown). These findings strongly confirmed the role of core a.a. 70 in disease progression, independent of any IFN response.

It is a focus of interest how the core sequence evolves with time or with the course of disease. If the core sequences were fixed throughout the course of disease, HCV with core 70Q might be an "oncogenic" virus, while HCV with core 70R might be "non-oncogenic", and the initial viral sequence might forecast future liver disease. In this study, we have demonstrated that core sequences changed in 15% (15/98) of patients during the observation period of around 10 years. Among these changes, R70Q ($N = 11$) was more common than Q70R ($N = 4$). Interestingly, changes in this region were significantly associated with disease activity or HCC development, although patients with R70Q substitutions were significantly more likely to have exacerbation of the disease and Q70R substitutions were associated with the stable disease. These results demonstrate that the core a.a. 70 residue is not fixed, but often changes with time during the course of disease in close association with disease progression and HCC development. Although the molecular mechanism of their interaction needs further exploration, this result highlights the important clinical and basic implications for the association between host and virus.

The importance of the IL28B SNP has been demonstrated recently in HCV infection in terms of a correlation with treatment outcome of pegylated-IFN plus ribavirin

therapy [16–19]. The contribution of the IL28B SNP to the outcome of therapy was confirmed in successive studies, although the mechanism remains under investigation. On this basis, we sought to investigate the impact of the IL28B SNP on disease progression and HCC development, separate from the IFN-based treatment response. As shown in Table 4, we compared the clinical features between the two groups (IL28B GG/TG vs. IL28B TT). Importantly, this comparison disclosed a significant correlation between the core a.a. 70 polymorphisms and the IL28B SNP ($p < 0.001$) and confirmed the existence of a complex interaction between the host and the virus in chronic HCV infection. According to the result, patients with IL28B TG/GG were more likely to be infected with HCV with core a.a. 70Q/H than with core a.a. 70R and vice versa. Although the molecular mechanisms of their relationship remain unknown, it could be speculated that the IL28B SNP has an influence on the viral core sequences, because the host IL28B SNP remains fixed and cannot be influenced by the viral core sequence.

On the other hand, we observed no evident association between the IL28B SNP and HCC development. This was rather unexpected because it is considered that the IL28B SNP has a significant influence on the core a.a. 70 residue. Therefore, to clarify the correlation among core a.a. 70, IL28B, and HCC development, we undertook multivariate analysis to extract the independent variables affecting the core 70 residue. As demonstrated in Table 5, the IL28B SNP and the development of HCC were extracted as variables independently correlated with the core a.a. 70 residue. The result indicates that the core a.a. 70 residue was not only influenced by the IL28B SNP, but also by factors strongly related to HCC development, independent of the IL28B SNP. When considering the result, it is not strange if there is no direct relationship between IL28B SNP and HCC development. In contrast, multivariate analysis undertaken for disclosing factors influencing HCC development revealed that core a.a. 70 residue was a variable independently associated with HCC development other than age, albumin, or platelets even though the IL28B SNP was not extracted (Table 6). However, further comprehensive studies are warranted to disclose the molecular mechanisms for the complicated relationships among core a.a. 70, IL28B, and HCC development.

In conclusion, we have shown that core a.a. 70 was closely associated with disease progression and, often, changes of that residue were accompanied by temporal changes in liver damage, in close relationship with the IL28B SNP.

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ORIGINAL ARTICLE

Characterization of naturally occurring protease inhibitor-resistance mutations in genotype 1b hepatitis C virus patients

Hiroko Shindo · Shinya Maekawa · Kazuki Komase · Ryota Sueki · Mika Miura · Makoto Kadokura · Kuniaki Shindo · Fumitake Amemiya · Takatoshi Kitamura · Yasuhiro Nakayama · Taisuke Inoue · Minoru Sakamoto · Shun-ichi Okada · Yasuhiro Asahina · Namiki Izumi · Masao Honda · Shuichi Kaneko · Nobuyuki Enomoto

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Abstract
Background and aims Protease inhibitor (PI)-resistant hepatitis C virus (HCV) variants may be present in substantial numbers in PI-untreated patients according to recent reports. However, influence of these viruses in the clinical course of chronic hepatitis C has not been well characterized.
Methods The dominant HCV nonstructural 3 (NS3) amino acid sequences were determined in 261 HCV genotype 1b-infected Japanese patients before pegylated interferon plus ribavirin (PEG-IFN/RBV) therapy, and investigated the patients' clinical characteristics as well as treatment responses including sustained virological response (SVR) rate. HCV-NS3 sequences were also determined in 39 non-SVR patients after completion of the therapy.
Results Four single mutations (T54S, Q80K, I153V, and D168E) known to confer PI resistance were found in 35 of 261 patients (13.4%), and double mutations (I153V plus T54S/D168E) were found in 6 patients (2.3%). Responses to PEG-IFN/RBV therapy did not differ between patients with and without PI-resistance mutations (mutation group, SVR 48%; wild-type group, SVR 40%; $P = 0.38$). On the other hand, two mutations appeared in two non-SVR patients after PEG-IFN/RBV therapy (I153V and E168D, 5.1%).
Conclusions PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. The impact of these mutations in the treatment of PIs is unclear, but clinicians should pay attention to avoid further development of PI resistance.

Keywords HCV · Protease inhibitor · Naturally occurring viral resistance mutations

Introduction

Hepatitis C virus (HCV) infects more than 170 million persons worldwide and thus represents a global health problem. At least 130 million infected individuals are chronic carriers of HCV and are at significant risk of developing liver cirrhosis and hepatocellular carcinoma [1]. The current standard treatment with pegylated interferon plus ribavirin (PEG-IFN/RBV) is complicated by frequent adverse reactions, and a sustained virologic response (SVR) can be achieved only in 50% of patients infected with the most prevalent genotype 1 [2]. In Japan, since 70% of patients are infected with intractable genotype 1b HCV, more effective treatments are urgently required.

A promising approach is the development of specifically targeted antiviral therapies for hepatitis C (STAT-C). HCV-specific protease inhibitors (PIs) target an essential step in HCV replication by blocking the nonstructural 3/4A (NS3/4A) protease-dependent cleavage of the HCV polyprotein

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H. Shindo · S. Maekawa (✉) · K. Komase · R. Sueki · M. Miura · M. Kadokura · K. Shindo · F. Amemiya · T. Kitamura · Y. Nakayama · T. Inoue · M. Sakamoto · S. Okada · N. Enomoto
First Department of Internal Medicine, University of Yamanashi, 1110, Shimokato, Chuo, Yamanashi 409-3898, Japan
e-mail: maekawa@yamanashi.ac.jp

Y. Asahina · N. Izumi
Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan

M. Honda · S. Kaneko
Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan

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[1]. Among these NS3/4A PIs, telaprevir, boceprevir, SCH446211, danoprevir (ITMN-191), nalaprevir (SCH900 518), and TMC435 are now under clinical trials [1, 3–7]. In PROVE1 and PROVE2 studies [3, 4] undertaken in North America and Europe, the SVR rate was favorable (67 and 69%, respectively) in a triple therapy regimen including telaprevir. In addition, some studies have suggested that shortening of treatment duration may be possible for patients who achieve a rapid virologic response (RVR) [8, 9].

However the sole use of STAT-C drugs, such as PIs, promotes production and selection of drug-resistant variants in patients experiencing viral rebound during treatment [3, 10, 11] as well as in HCV replicon experiments [11, 12]. Therefore, these drugs should be used in combination with the PEG-IFN/RBV to prevent the appearance of drug-resistant variants. However, Kuntzen et al. [13] demonstrated the presence of these drug-resistant variants in high frequencies (8.6–16.2%) by population-based sequencing in patients not treated with the drugs [1, 13]. Gaudieri et al. [14] have suggested that regions of NS3 protease and NS5B polymerase are likely to be under HLA immune pressure and therapeutic selection, and that drug-resistant variants may occur naturally to escape the immune system. These observations seem quite astonishing and troubling, since a substantial number of patients may not respond to the new therapies such as STAT-C drugs.

In the present study, to assess the prevalence of NS3 mutations conferring PI resistance in HCV genotype 1b-infected Japanese patients who had not been previously treated with PIs, as well as to assess the influence of those mutations in response to PEG-IFN/RBV therapy, the dominant HCV-NS3 sequences were determined in 261 HCV-1b patients before starting the PEG-IFN/RBV therapy.

Methods

Patients

Serum samples were acquired from 261 HCV genotype 1b-infected adult Japanese patients before combination therapy with PEG-IFN (PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2004 and 2008 at the University of Yamanashi, Musashino Red Cross Hospital and Kanazawa University. The therapy was administered according to the standard PEG-IFN/RBV treatment protocol established for Japanese patients by a hepatitis study group of the Ministry of Health, Labor, and Welfare, Japan. Specifically, the patients were subcutaneously administered PEG-IFNα-2b, 1.5 μg/kg body weight, once weekly and RBV 600–800 mg daily per os for 48 weeks. These patients were not infected with human immunodeficiency virus (HIV). The study was approved by the ethics committees of all participating universities and the hospital, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board at Massachusetts General Hospital. Written informed consent was obtained from each study participant.

Amplification and sequencing of full-length HCV genomes

Viral loads were determined using the Amplicor HCV RNA kit, version 2.0 (Roche Diagnostics, Tokyo, Japan) or the Cobas TaqMan test (Roche Diagnostics). HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the manufacturer's protocol. Complementary DNA was synthesised using Superscript II (Invitrogen, Tokyo, Japan) and random primers (Invitrogen), and then amplified by two-step nested PCR using the primers listed in Supplementary Table 1. All samples were initially denatured at 95°C for 7 min, followed by 40 cycles of amplification with denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s using the BD Advantage™ 2 PCR Enzyme system (BD Biosciences Clontech, CA, USA). PCR amplicons were directly sequenced using BigDye Terminator version 3.1 (ABI, Tokyo, Japan) and universal M13 forward/reverse primers using an ABI prism 3130 sequencer (ABI).

Sequence alignment and analysis

Sequences were determined in both directions, particularly for the ambiguous stretches, were assembled using the Vector NTI software (Invitrogen), and base-calling errors were corrected following the inspection of chromatograms. If mixed bases were detected as two different chromatogram peaks at the same residue, only the dominant base was called after evaluation of all overlapping fragments. A consensus sequence was generated from the alignment on the basis of the most common amino acid at each site.

Determination of PI resistance mutations

Multiple viral NS3 mutations were observed in amino acid positions reported to confer PI resistance among 261 patients: V36, Q41, F43, T54, V55, Q80, R109, I153, R155, A156, D168, V170, and M175. NS3 amino acid mutations with proven PI resistance in previously published studies (Table 1) were designated as resistance proven mutations (e.g., V36M/A). Mutations in the PI-resistance site not known to confer drug resistance were designated resistance unproven mutations (e.g., V36I). Patients were allocated to two groups according to the presence of PI-resistance

mutations (including resistance unproven mutations), and clinical characteristics including HCV RNA levels and responses to PEG-IFN/RBV therapy were compared. To assess the influence of PEG-IFN/RBV therapy on NS3 mutational status, posttreatment HCV-NS3 sequences in 39 of 58 non-SVR patients were also examined.

Statistical analysis

Statistical differences in the data, including all available patients' demographic, biochemic, hematologic, and virologic data such as sequence variation factors, were determined among the various groups by Student's *t* test or Mann–Whitney *U* test for numerical variables and Fisher's exact probability test for categorical variables.

Results

Prevalence of dominant PI-resistance-associated nonstructural 3 mutations in untreated patients

Figure 1 shows the frequency of substitutions in 261 patients for each of 181 NS3 protease amino acid residues compared to the consensus sequence. A total of 41 resistance proven mutations were detected in 35 (13.4%) patients: T54S (14 patients, 5.4%), Q80K (1 patient, 0.4%), I153V (22 patients, 8.4%), D168E (4 patients, 1.5%), T54S plus I153V double mutation (4 patients, 1.5%), and I153V plus D168E double mutation (2 patients, 0.8%). The mutation number increased to 54 in 47 (18.0%) patients when resistance unproven mutations were included: V36I (2 patients, 0.8%), I153L (11 patients, 4.2%), and I153V plus V36I double mutation (2 patients, 1.5%). Double mutations were found in 7 patients (2.7%) (Table 1). Q80L was observed in 47 (18%) patients but these were excluded from consideration because a previous study demonstrated that this mutation does not confer resistance [15]. All mutations observed in this study would confer low- to moderate-level PI resistance according to previous studies [6, 15–19]. No mutations conferring high-level resistance such as R155 or A156 [11, 17, 19–22] were observed.

Clinical characteristics of patients with PI-resistance mutations

Table 2 presents the characteristics of patients classified according to the presence of PI-resistance mutations

Table 1 Prevalence of PI-resistance-associated NS3 mutations

Drug-resistance mutations described in the literature				Detected resistance mutations
NS3 residue	Resistance mutations	Drugs	References	Genotype 1b ($N = 261$), (%)
V36	A, M, L, G, C	Telaprevir, Boceprevir	[1, 3, 4, 10, 11, 19, 31, 37]	I × 2 (0.8)
Q41	R	ITMN-191, Boceprevir	[19]	
F43	S, C	ITMN-191, Boceprevir, Telaprevir, TMC435	[15, 19]	
T54	A, S	Telaprevir, Boceprevir, SCH900518	[1, 3, 10, 11, 19, 20, 31, 38]	**S × 14 (5.4)**
V55	A	Boceprevir	[1]	
Q80	R, K	TMC435	[6, 15]	**K × 1 (0.4)**
R109	K	SCH446211	[17]	
I153	V	SCH446211	[17]	**V × 22 (8.4)**, L ×11 (4.2)
R155	K, T, I, M, G, L, S, Q	Telaprevir, Boceprevir, ITMN-191, BILN2061, TMC435	[1, 3, 4, 6, 10, 11, 15, 19, 20]	
A156	S, T, V, I, G	Telaprevir, Boceprevir, ITMN-191, BILN2061, SCH446211, TMC435, SCH900518	[1, 3, 4, 10, 11, 15, 17, 19, 20, 38]	
D168	A, V, E, N, T, H	BILN2061, ITMN-191, TMC435	[6, 15, 20]	**E × 4 (1.5)**
V170	A	Telaprevir, Boceprevir	[1, 19, 20]	
M175	L	Boceprevir	[39]	
Total number (%) of patients with resistance proven mutations				35 (13.4)
Total number (%) of patients with resistance proven and unproven mutations				47 (18.0)

Amino acid mutations conferring PI resistance in the literatures and those observed in PI-treatment-naive patients in this study are indicated. Bold indicates resistance proven mutations, and the others indicate resistance unproven mutations

Double mutations found were as follows: V36I and I153V × 1, T54S and I153V × 4, I153V and D168E × 2

(including resistance unproven mutations). Age, sex ratio, body mass index, alanine aminotransferase (ALT) levels, serum albumin, platelet count, and fibrosis stage did not differ between the NS3 mutation and wild-type groups. No significant difference was observed between the two groups in the parameters of PEG-IFN/RBV treatment response, HCV sequence variations in interferon sensitivity determining region (ISDR), Core 70, interferon plus ribavirin resistance-determining region (IRRDR), or interleukin 28B (IL28B) single nucleotide polymorphism (SNP) (rs8099917; T/G and G/G vs. T/T) [23–30]. These clinical variables were also compared between the mutation group defined as resistance proven mutations and the wild-type group, but no notable differences were observed.

Unimpaired in vivo fitness of viral strains with resistance mutations

Because most PI-resistance mutations described till date have been associated with reduced replicative capacity of varying degrees [1, 10, 11, 13, 17, 20–22, 31, 32], we examined viral replication levels in patients with drug-resistance mutations (Fig. 2). The estimated P value indicated no significant difference between the mutation (median 1,500 KIU/ml) and wild-type (median 1,800 KIU/ml) groups ($P = 0.69$). The results indicate that drug-resistant HCVs were not necessarily impaired in their ability to replicate in vivo. However, patients with double mutations ($N = 7$) tended to have low viral loads (median 1,200 KIU/ml) ($P = 0.09$).

Resistance mutations and virologic response to PEG-IFN/RBV therapy

To determine the difference in virologic response to PEG-IFN/RBV therapy according to the PI mutation, frequency of HCV RNA levels below detection at 4 weeks (rapid viral response, RVR) and 12 weeks (complete early viral response, cEVR), and SVR rate (%) were investigated in each group. The frequency of HCV RNA levels below detection at 4 and 12 weeks was 14 and 50%, respectively, in the mutation group, and was 11 and 46%, respectively, in the wild-type group. The SVR rate was 48 and 40% in the mutation and wild-type groups, respectively ($P = 0.38$). No significant difference was observed between the two groups in any of the indexes investigated (Table 2). The time-dependent viral clearance rate during PEG-IFN/RBV therapy was estimated in 133 patients including 25 patients (19%) with PI-resistance mutations available for the analysis. Kaplan–Meier analysis demonstrated that HCV clearance did not differ between the two groups with and without resistance mutations (log-rank test, $P = 0.30$) (Fig. 3).

Changes in nonstructural 3 amino acid sequence diversity during PEG-IFN/RBV therapy

Full-length NS3 protease sequences were determined in 39 non-SVR patients after PEG-IFN/RBV therapy. A single amino acid change at resistance-associated sites in two patients was observed. In one patient, isoleucine (Ile) at position 153 changed to valine (Val), and glutamic acid (Glu) changed to aspartic acid (Asp) at position 168 in the second (Fig. 4). At the nucleotide level, ATC (Ile) changed to GTC (Val) in I153V, and GAA (Glu) changed to GAC (Asp) in E168D. Both mutations were caused by one nucleotide exchange. No other changes were observed in the other 37 patients.

Discussion

Here we report that in 18% (47/261) HCV genotype 1b-infected patients who had not been previously treated with NS3 PIs, the viral genome contained dominant amino acid mutations within the NS3 PI-resistance sites. Even after confining the data to established PI-resistance mutations, the mutation rate was still significant in 13.4% (35/261). No clinical differences were observed between patients

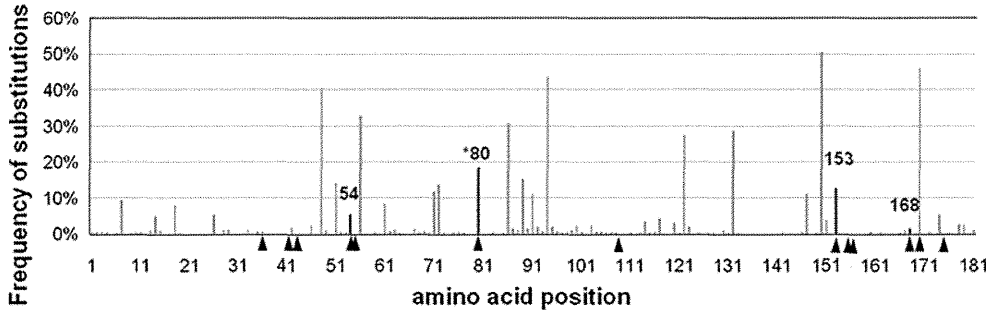

Fig. 1 Frequency of polymorphic mutations for each of the 181 NS3 protease amino acid residues in 261 patients. *Arrowheads* indicate the sites reported to confer PI resistance. *Dark bars* denote the amino acid variations at the resistant sites in this study. **80*, we detected one resistant mutation (Q80K) and 47 (18%) non-resistant variations (Q80L) at the 80th residue

Table 2 Characteristics of patients with or without HCV genomes harboring drug-resistance mutations

Characteristics	Mutation type ($N = 47$)	Wild-type ($N = 214$)	P value
Patients' characteristics			
Age, median (range)	59 (46–72)	57 (19–77)	0.17
Male, no. (%)	26 (55)	112 (52)	0.70
BMI, median (range)	23.2 (15.5–31.9)	22.8 (16.1–31.9)	0.41
ALT IU/ml	81.3 ± 72.6[a]	74.8 ± 51.9	0.93
Serum albumin g/dl	4.00 ± 0.37	4.01 ± 0.36	0.81
Platelet count × 10^4/μl	15.8 ± 4.3	14.5 ± 4.8	0.18
HCV RNA KIU/ml, median (range)	1,500 (58–6,310)	1800 (28–15,849)	0.69
Fibrosis, no. (%)			0.97
F0	0 (0)	7 (3)	
F1	23 (50)	89 (42)	
F2	9 (20)	52 (24)	
F3	9 (20)	40 (19)	
F4	5 (11)	26 (12)	
IFN pre-treatment no. (%)	15/40 (38)[b]	66/172 (38)	1.00
IL28B (rs8099917) T/G or G/G no. (%)	6/20 (30)	19/67 (28)	1.00
Response to PEG-IFN/RBV therapy			
SVR total cases no. (%)	22/46 (48)	83/210 (40)	0.38
RVR in total cases no. (%)	6/44 (14)	22/195 (11)	0.83
cEVR in total cases no.(%)	22/44 (50)	92/200 (46)	0.75
SVR 48w treatment no. (%)	16/29 (55)	55/130 (42)	0.29
End of treatment response no. (%)	26/41 (63)	123/202 (61)	0.91
HCV genome sequence variation			
ISDR mutation ≤1 no. (%)	32/46 (70)	167/210 (80)	0.21
Core70 R no. (%)	26/44 (59)	136/210 (65)	0.56
IRRDR mutation >3 no. (%)	25/38 (66)	107/190 (56)	0.34

[a] Mean ± SD

[b] Number/total number (%)

harboring viruses with and without these mutations. Moreover, no differences were observed in the responses of either group to PEG-IFN/RBV therapy.

Recent studies reported that significant number of patients who were never treated with PI possess viral sequences with PI-resistance-associated NS3 mutations. In these studies, the prevalence of PI-resistance mutations was determined to be 8.6–16.2% [13, 14], in HCV genotype 1- and 3-infected patients in European–American populations. These patients were often coinfected with HIV. Analysis of the public HCV databases (EuHCVdb and Los Alamos) also reported the presence of naturally occurring PI-resistance-associated NS3 mutations in worldwide isolates [33]. However, in vivo and in vitro studies demonstrated that most of the mutations observed conferred only low- to moderate-level PI resistance [7, 13, 14, 34, 35]. Regarding viral fitness, PI-resistant HCVs show lower fitness at varying degrees as revealed by in vitro studies [1, 10, 11, 17, 20–22, 31, 32], but HCV RNA levels in a clinical study did not differ significantly. The response to PEG-IFN/RBV therapy was almost comparable to that in HCV-infected patients without PI-resistance mutations either in HCV replicon experiments or in a clinical study of small number of treated patients [34].

The prevalence of 13.4% for PI-resistance-proven patients observed in the present study was almost comparable to the results of previous studies. Although HIV is known to increase HCV replication in coinfection with HCV [36], and HIV patients are often treated with the HIV-specific PIs, the HIV infection might not affect the natural occurrence of HCV-specific PI-resistance mutations since our studied patients were all proven to be free from coinfection with HIV infection. As shown in Table 1 and Fig. 1, I153 V (22/261, 8.4%), T54S (14/261, 5.4%), and D168E (4/261, 1.5%) were among the most prevalent PI-resistance-proven mutations in the present study. The most frequent mutation detected in our study I153V was reported to appear secondarily to the occurrence of R109K mutations in a HCV replicon system [17]. Although the role of this mutation is not understood, the I153V mutation on its own conferred SCH446211 resistance to the HCV replicon to a lesser degree [17]. Interestingly, I153V was often found in double mutations in our study, as shown in Fig. 2. This suggests analogy between in vitro and in vivo data. T54S and D168E, the other frequent mutations, have been also reported to occur as single dominant mutations in previous in vitro or in vivo studies in HCV genotype 1

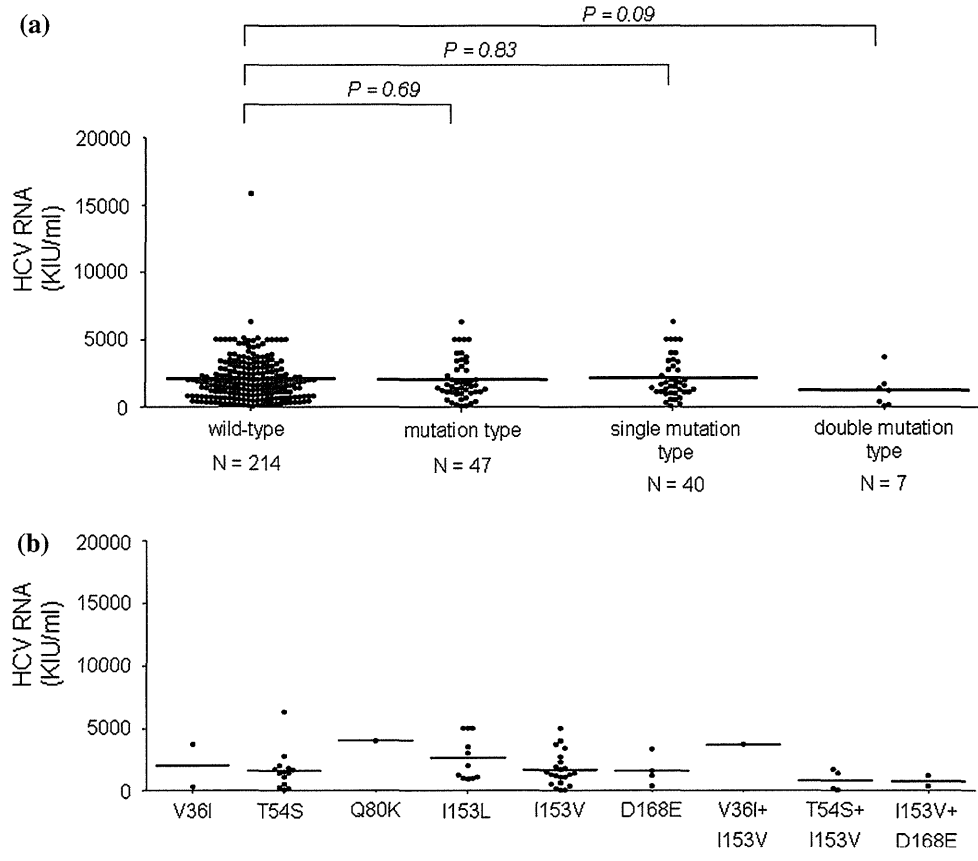

Fig. 2 In vivo fitness of HCV with PI-resistance-associated NS3 mutations. HCV RNA levels were compared between patients with and without NS3 PI-resistance-associated mutations (**a**) and between patients with each resistance mutation (**b**). The estimated *P* value (Mann–Whitney *U* test) indicates no significant difference between the wild-type and other groups (wild-type vs. mutation type, wild-type vs. single mutation type, and wild-type vs. double mutation type). (Wild-type, $N = 214$; mutation type, $N = 47$; single mutation type, $N = 40$; double mutation type, $N = 7$; V36I, $N = 2$; T54S, $N = 14$; Q80K, $N = 1$; I153L, $N = 11$; I153V, $N = 22$; D168E, $N = 4$; E176A, $N = 1$; V36I + I153V, $N = 1$; T54S + I153V, $N = 4$, and I153V + D168E, $N = 2$)

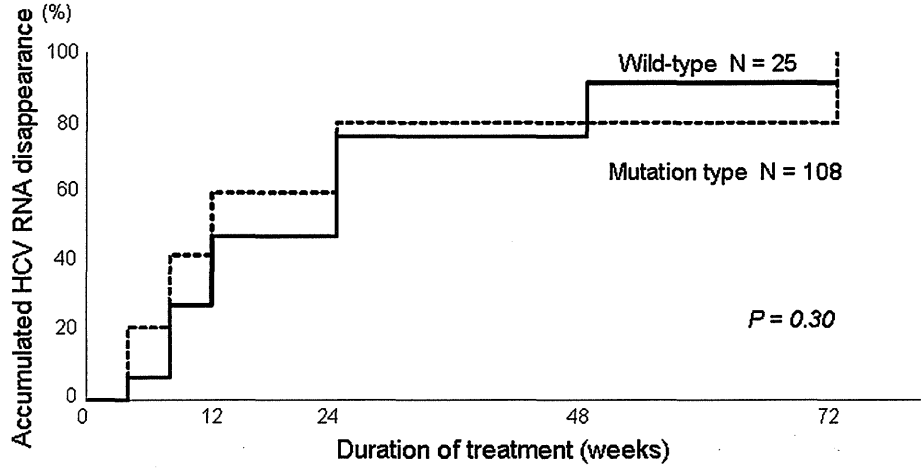

Fig. 3 Comparison of virologic response to PEG-IFN/RBV therapy between HCV-infected patients with and without PI-resistance-associated NS3 mutations. Time-dependent HCV clearance rate analysis was based on serum HCV RNA positivity during PEG-IFN/RBV therapy for HCV isolates with resistance mutations or wild-type sequences. A total of 133 patients for whom the limit of viral genome detection could be determined were analyzed. Among this group, NS3 mutations were detected in 25 patients (19%). The estimated *P* value (log-rank test) shows no significant difference between the two groups ($P = 0.30$)

infections showing moderate degrees of resistance [16, 18, 19].

Most PI-resistance mutations described to date have been associated with varying degrees of reduced replicative capacity [10, 11, 17, 20–22, 31, 32]. In the present study, HCV RNA levels of those patients with low- to moderate-level resistance mutations were similar to those in patients in the wild-type groups, suggesting that in vitro viral fitness

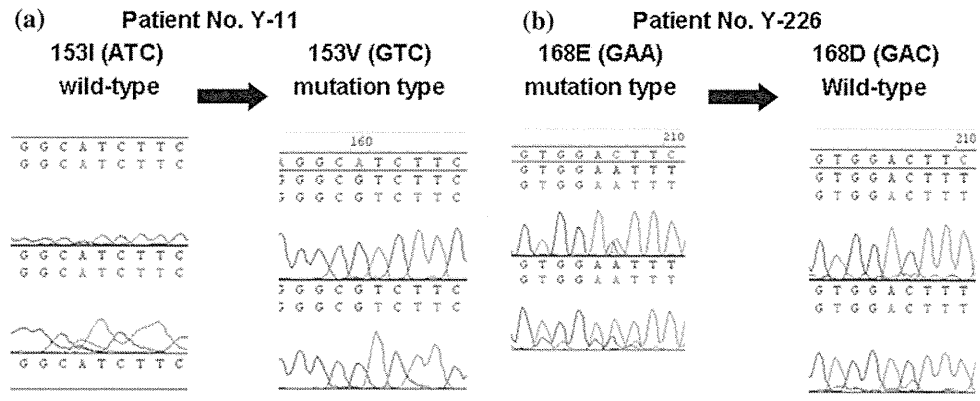

Fig. 4 Appearance of PI-resistance-associated NS3 mutations during the PEG-IFN/RBV therapy. Chromatograms show part of the HCV NS3 sequence demonstrating PI-resistance mutations in two patients receiving therapy. **a** Site 153 isoleucine (Ile) (ATC) changed to valine (Val) (GTC), **b** Site 168 glutamic acid (Glu) (GAA) changed to aspartic acid (Asp) (GAC)

does not necessarily reflect in vivo viral fitness. This, however, does not rule out the possibility that some unknown compensatory viral mutations might have resulted in upregulation of reduced viral fitness. Interestingly, although the replicative capacity conferred by a single mutation seemed to be the same, the HCV RNA levels of double mutations were frequently low, suggesting that double mutations might weaken viral fitness.

In previous studies, clinical characteristics representing the state of liver disease other than HCV RNA levels were not studied in patients with PI-resistance mutations. In this study, we show that those clinical characteristics did not differ according to the presence of viral NS3 mutations. As shown in Table 2, age, sex ratio, fibrosis stage, ALT levels, serum albumin, platelet count, and past history of IFN pretreatment did not differ according to the presence of NS3 mutations. These results suggest that NS3 mutations occur independently of disease progression. Moreover, no evident differences were observed between viral and host factors known to affect IFN-based treatment responses. However, viral amino acid variations in the core and NS5A or the allelic frequency of IL28B SNPs, which were recently reported for the close relationship of responses to PEG-IFN/RBV therapy, did not differ between the two groups.

A significant outcome of the present study is the demonstration that PI-resistance mutations might not affect responses to PEG-IFN/RBV therapy. Previous in vitro studies demonstrated that HCV replicons harboring PI-resistance mutations were also sensitive to IFN treatment [31]. In addition, recent clinical studies also indicated that PI-resistance mutations were sensitive to the PEG-IFN/RBV [10, 34]. However, our analysis was more comprehensive because viral and host factors that contribute to treatment responses were simultaneously analyzed. A unique aspect of the present study is that we investigated the influence of the PEG-IFN/RBV treatment on the occurrence of new PI mutations by direct nucleotide sequencing, and were able to show that the PEG-IFN/RBV might not induce amino acid mutations.

Will the pre-existence of naturally occurring PI-resistance mutations have an influence on future treatment of HCV infections? Since new PIs are on the verge of clinical use, all clinicians should bear in mind the substantial numbers of HCV-infected patients with PI-resistance mutations. Although the degree of resistance is considered to be low or moderate in untreated patients, weak resistance might progress to more potent resistance with additional mutations, when PIs become widely used. Therefore, all clinicians need to be sufficiently prepared for the possibility of later onset of PI-resistance mutations that confer greater drug resistance and concomitant poorer responses to therapy. In SPRINT-1 study, the lead-in therapy was associated with a modestly lower rate of breakthrough than with no lead in [7]. Considering that PEG-IFN/RBV was equally effective for PI-resistant viruses, sufficient "lead-in" therapy before the administration of PIs could be an option in the forthcoming triple therapy modality.

In conclusion, we demonstrate here that PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. Although the degree of resistance might not be strong, clinicians will need to consider this upon the introduction of triple therapy.

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Analysis of the Complete Open Reading Frame of Genotype 2b Hepatitis C Virus in Association with the Response to Peginterferon and Ribavirin Therapy

Makoto Kadokura[1☯], Shinya Maekawa[1*☯], Ryota Sueki[1], Mika Miura[1], Kazuki Komase[1], Hiroko Shindo[1], Fumitake Amemiya[1], Tomoyoshi Uetake[1], Taisuke Inoue[1], Minoru Sakamoto[1], Mina Nakagawa[2], Naoya Sakamoto[2], Mamoru Watanabe[2], Nobuyuki Enomoto[1]

1 First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Chuo, Yamanashi, Japan, **2** Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Bunkyo, Tokyo, Japan

Abstract

Background and Aims: Patients infected with genotype 2b hepatitis C virus (HCV) generally can achieve favorable responses to pegylated-interferon plus ribavirin therapy (PEG-IFN/RBV). However, a proportion of patients show poorer responses and the correlation between viral sequence variation and treatment outcome remains unclear.

Methods: The pretreatment complete open reading frame (ORF) sequences of genotype 2b HCV determined by direct sequencing were investigated for correlation with the final outcome in a total of 60 patients.

Results: In this study group, 87.5% (14/16) of non-sustained virological response (non-SVR) patients (n = 16) were relapsers. Compared to sustained virological response (SVR) patients (n = 44), non-SVR patients were older and could not achieve prompt viral clearance after the therapy induction. Comparing each viral protein between the two groups, viral sequences were more diverse in SVR patients and that diversity was found primarily in the E1, p7, and NS5A proteins. In searching for specific viral regions associated with the final outcome, several regions in E2, p7, NS2, NS5A, and NS5B were extracted. Among these regions, part of the interferon sensitivity determining region (ISDR) was included. In these regions, amino acid substitutions were associated with the final outcome in an incremental manner, depending upon the number of substitutions.

Conclusions: Viral sequences are more diverse in SVR patients than non-SVR patients receiving PEG-IFN/RBV therapy for genotype-2b HCV infection. Through systematic comparison of viral sequences, several specific regions, including part of the ISDR, were extracted as having significant correlation with the final outcome.

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* E-mail: maekawa@yamanashi.ac.jp

☯ These authors contributed equally to this work.

Introduction

Worldwide, 180 million people are estimated to be infected with hepatitis C virus (HCV), a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. In HCV-infected patients with chronic hepatitis, treatment with interferon (IFN)-based therapy can result in viral clearance as well as biochemical and histological improvements [2]. In this IFN-based therapy, HCV genotype is the most significant factor affecting treatment responses [3,4].

In genotype 2b HCV infection, 80% of patients with high viral titers can achieve a sustained virological response (SVR) to the regimen of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) for 24 weeks [5,6]. This response is high considering that much lower percentages of patients infected with other genotypes can achieve SVR, especially with genotype 1 [1]. However, in other words, 20% of patients infected with genotype 2b HCV still cannot clear the virus and remain at risk of developing HCC. On the other hand, although various studies have been undertaken to clarify the factors contributing to the response to IFN-based therapy in genotype 1 infection, it remains poorly understood which patients with genotype 2b HCV infection will show unfavorable responses. Recently, the significance of IL28B single nucleotide polymorphisms (SNPs) in determining the response to PEG-IFN/RBV therapy was demonstrated in genotype 1 HCV infection [7,8]. However, the significance of IL28B SNPs was rather weak in genotype 2 HCV infection [9].

In terms of the association between HCV sequence variation and treatment responses, previous studies have reported that

Table 1. Baseline Characteristics of Studied Patients.

Characteristic	SVR (n = 44)	non-SVR (n = 16)	*P* value
Gender (Male/Female)	26/18	9/7	NS†
Age (yrs)	56 (22–72)*	59 (30–80)	0.04‡
BMI	23.5 (16.6–30.3)	24.7 (18.5–31.7)	NS‡
ALT (IU/l)	51 (19–380)	41 (17–390)	NS‡
GGTP (IU/l)	36 (11–133)	40 (17–292)	NS‡
T.Chol (mg/dl)	169 (119–225)	178 (145–217)	NS‡
WBC (/μl)	4600 (2620–7200)	5080 (3270–8600)	NS‡
Hb (g/dl)	14.2 (11.5–17.3)	14.6 (11.8–16.4)	NS‡
Platelet ($\times 10^4/mm^3$)	19 (7.1–31.8)	17.8 (8–36.7)	NS‡
Fibrosis score (0–2/≥3)§	38/5	7/3	NS†
HCV RNA (KIU/ml)	2050 (100–16000)	1800 (140–6300)	NS‡
IFN dose (≥80%/60–80%)	36/8	13/3	NS†
Ribavirin dose (≥80%/60–80%)	32/12	10/6	NS†
RVR rate (%)	55.8	6.3	0.0008†
EVR rate (%)	97.7	68.8	0.004†
ETR rate (%)	100	87.5	NS†

§: SVR : n = 43, non-SVR : n = 10.
*: median (range).
†: Fisher's exact probability test.
‡: Mann-Whitney's U test.
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amino acid variation in the NS5A-ISDR [10], NS5A-IRRDR [11], NS5B [12], PKR-eIF2 phosphorylation homology domain (PePHD) of E2 [13], and Core [14] correlate with the clinical outcome of IFN-based therapy, including PEG-IFN/RBV therapy for genotype 1b HCV infection. In the meantime, these viral sequence studies have been controversial regarding their true clinical importance, because the results of different studies were not always coincident [15,16,17]. On this background, recent studies trying to analyze the correlation of complete HCV open reading frame diversity, clinical characteristics, and the response to PEG-IFN/RBV therapy for genotype 1 HCV infection, in the most comprehensive approach yet attempted, have clarified that viral amino acid variation is associated with treatment responses, with consideration of racial background [18,19]. In genotype 2 infection, however, only a few studies have investigated the association of HCV sequence variation and treatment response [20,21] and the clinical significance has been yet established. We reported recently that variation of amino acid (aa) 110 in Core and amino acids (aa) 2258–2308 in NS5A were significantly associated with treatment outcome of the PEG-IFN/RBV therapy for genotype 2a HCV infection, through the analysis of the complete HCV ORFs in Japanese patients [22].

In this study, to assess comprehensively the influence of viral sequence variation on the response to the PEG-IFN/RBV therapy in genotype 2b HCV infection, we determined the complete pretreatment HCV ORFs from Japanese patients and investigated amino acid variation and its correlation with the response to combination therapy with PEG-IFN plus RBV.

Methods

Patients

A total of 77 adult Japanese patients infected with genotype 2b HCV, who received the combination therapy with PEG-IFN

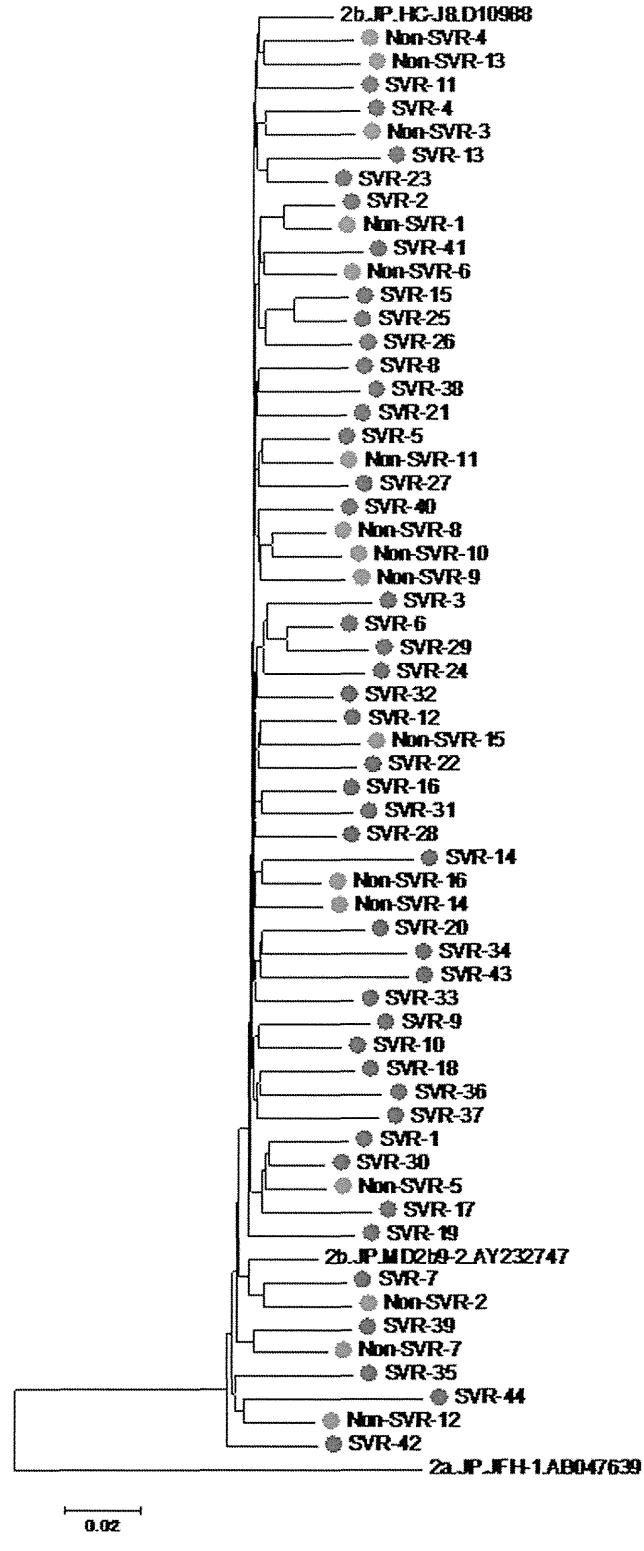

Figure 1. Phylogenetic analysis of the genotype-2b polyprotein sequences. In order to perform the phylogenetic analysis, we first aligned all 60 HCV complete ORF amino acid sequences obtained from the patients along with reference sequences (2b.HC-J8.D10988, 2.JP.MD2b9-2, and 2a.JP.JFH-1.AB047639), using the ClustalW program, and constructed the phylogenetic tree using the Neighbor-Joining method with MEGA version 4 software. Blue circles indicate SVR patients and red circles indicate non-SVR patients.
doi:10.1371/journal.pone.0024514.g001

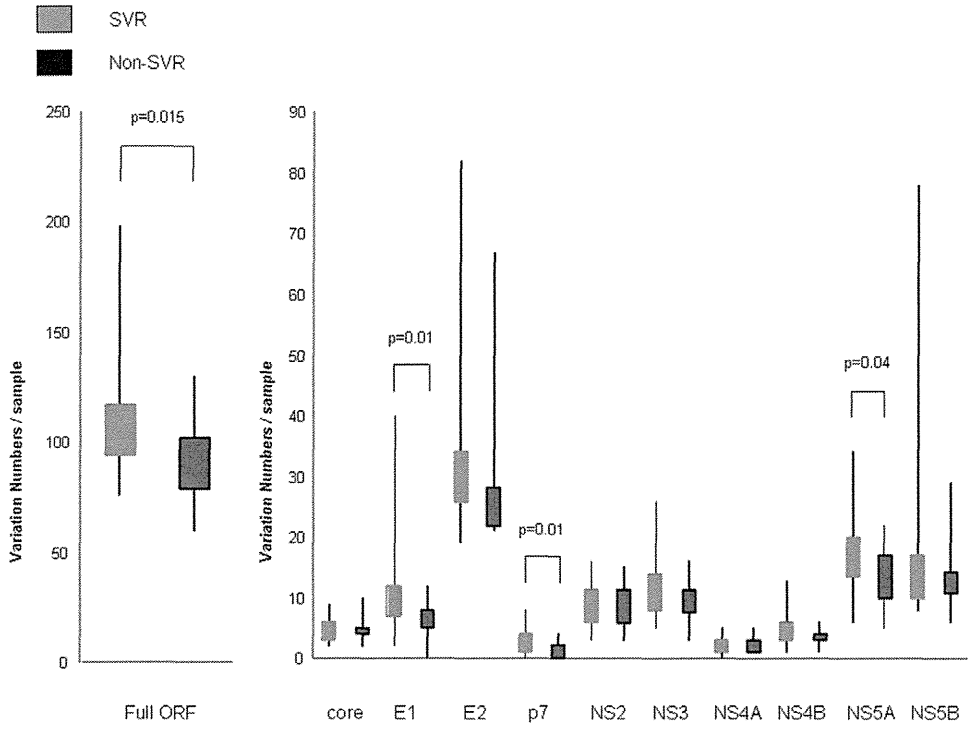

Figure 2. Number of amino acid substitutions per sample in the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) group. The numbers of variations, relative to a population consensus, that were unique to either SVR or non-SVR patients are shown for the complete open reading frame (ORF) (Fig. 1, left) and for each HCV protein (Fig. 1, right).
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(PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2005 and 2009 at University of Yamanashi, Tokyo Medical and Dental University, and related institutions were first included in the study. They all fulfilled following criteria: (1) negative for hepatitis B surface antigen, (2) high viral load (≥100 KIU/ml), (3) absence of

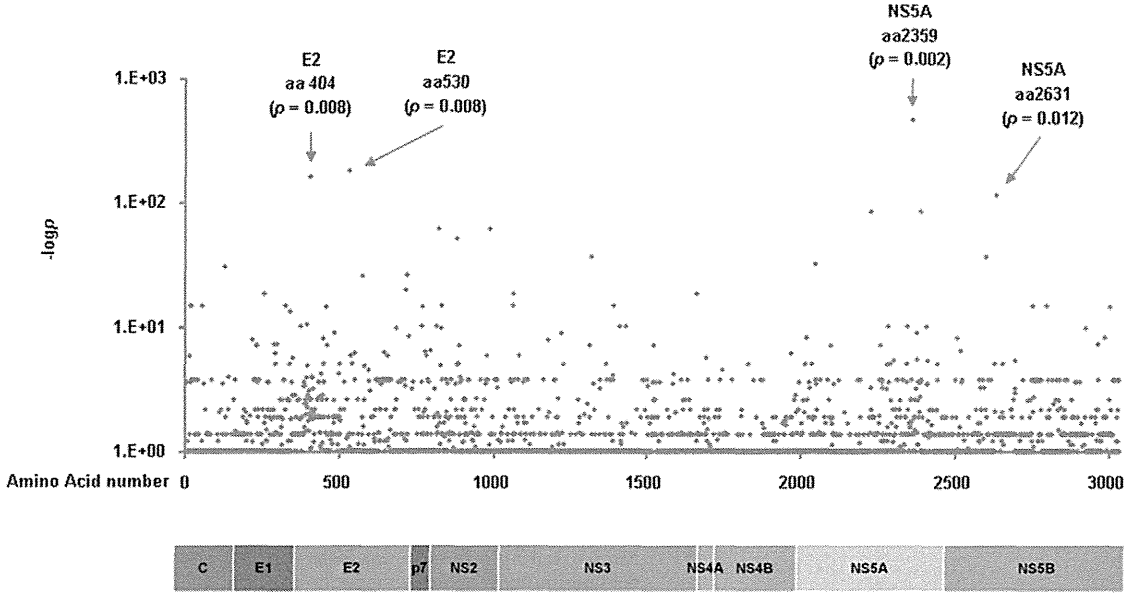

Figure 3. Different amino acid usage at each viral amino acid position between the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) patients. (a) Different amino acid usage at each viral amino acid position between the SVR and the non-SVR patients was analyzed by Fisher's exact probability test. The longitudinal axis shows the −logP value. (b) Sequence alignment in the Core region is demonstrated. Dashes indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes.
doi:10.1371/journal.pone.0024514.g003

Table 2. Variation at each Amino Acid Position and SVR rate.

	E2 aa 404 non T	E2 aa 530 non T	NS5A aa 2359 N	NS5B aa 2631 non P
SVR rate	86.1% (31*/36**, p = 0.008)	87.9% (29/33, p = 0.008)	82% (41/50, p = 0.002)	94.7% (18/19, p = 0.012)

*SVR number in patients fulfilling the criteria.
**Number of patients fulfilling the criteria.
doi:10.1371/journal.pone.0024514.t002

hepatocellular carcinoma, (4) no other form of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease, (5) free of co-infection with human immunodeficiency virus. To clearly disclose the non-SVR viral characteristics, we have considered only those patients who achieved total drug administration of 60% or more for both PEG-IFN and RBV, with the completion of the standard treatment duration. Moreover, although we excluded patients with extended therapy to make the studied population uniform, we have included non-SVR patients with extended therapy to clarify the specific characteristics of non-SVR patients, a minor population group. As a result, 17 patients were excluded for the following reasons: 1 patient received insufficient dose, 4 patients were discontinued from the therapy within 12 weeks, and 12 SVR patients received extended therapy. Finally, 60 patients were considered as eligible for the study. During the combination therapy, blood samples were obtained at least once every month before, during and after treatment and were analyzed for blood count, ALT and HCV RNA levels. Liver biopsy specimens were obtained from most of the patients. All patients gave written informed consent to the study. The study was approved by the ethics committees of University of Yamanashi, Tokyo Medical and Dental University, and related institutions. The therapy was performed according to the standard treatment protocol of PEG-IFN/RBV therapy for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan (PEG-IFNα-2b 1.5 μg/kg body weight, once weekly subcutaneously, and RBV 600–800 mg daily per os for 24 weeks).

Complete HCV-ORF Sequence Determination by Direct Sequencing from Pretreatment Sera

HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the following protocol. Briefly, 150 μl of serum were mixed with 700 μl of Isogen, and an aqueous phase was extracted with 150 μl of chloroform. RNA was precipitated with 600 μl of isopropanol and with 2 μl of Glyco Blue (Ambion, Tokyo, Japan) as a carrier. The purified RNA was washed once with ethanol and finally dissolved in 15 μl of distilled water and stored at −70°C until use.

Complementary DNA was synthesized according to the following protocol. 30 μl of the reverse transcription mixture were adjusted to contain 3 μl of the RNA solution, 300 U of Superscript

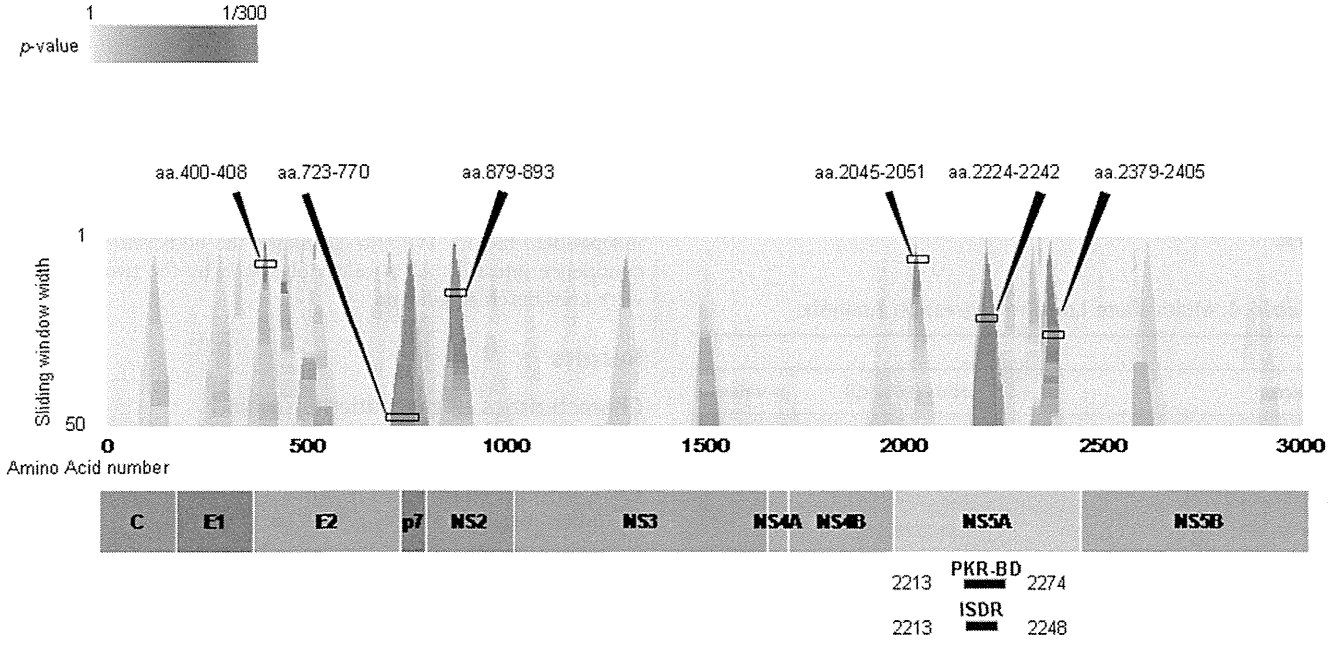

Figure 4. Sliding window analysis. (a) Comparison of amino acid variation between the SVR and non-SVR patients across HCV "regions" using sliding window analysis was performed. Viral regions affecting treatment outcome are shown as red areas. There are six hot areas: amino acid 400–408 and 723–770 in the E2 region, amino acid 879–893 in the NS2 region and, amino acid 2045–2051, 2224–2242 and 2379–2405 in the NS5A region. (b) Sequence alignment in the nonstructural (NS)5A around amino acids 2213 to 2274 is demonstrated. Dashes indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes.
doi:10.1371/journal.pone.0024514.g004

Table 3. Number of Amino Acid Substitutions in each Region and SVR rate.

	E2 aa 400–408 mutation ≥4	E2 aa 723–770 mutation ≥2	NS2 aa 879–893 mutation ≥2	NS5A aa 2045–2051 absense of mutation	NS5A ISDR (aa 2213–2248) mutation ≥1	NS5A aa 2224–2242 mutation ≥1	NS5A aa 2379–2405 mutation ≥2
SVR rate	86.5% (32*/37**) p = 0.006	100% (18/18) p = 0.001	94.7% (18/19) p = 0.01	89.7% (35/39) p = 0.0002	86.1% (31/36) p = 0.008	90.9% (30/33) p = 0.001	90.9% (20/22) p = 0.03

*SVR number in patients fulfilling the criteria.
**Number of patients fulfilling the criteria.
doi:10.1371/journal.pone.0024514.t003

II (Invitrogen, Tokyo, Japan) with an accompanied buffer according to the manufacturer's instructions, 60 units of RNase inhibitor (Promega Corp., Madison, WI), and 300 pg of random primers (Invitrogen). The mixture was incubated at 37°C for 30 min. The HCV genome was amplified with 24 partially overlapping primer (Table S6) sets, designed specifically for this study, to perform two-step nested PCR. As previously reported, a M13 forward primer (5′-TGTAAAACGACGGCCAGT-3′) and a M13 reverse primer (5′-CAGGAAACAGCTATGACC-3′) were attached to the 5′ termini of the sense and antisense second-round PCR primers, respectively, to facilitate direct sequencing. All samples were initially denatured at 95°C for 7 min., followed by 40 cycles with denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 45 seconds with BD Advantage™ 2 PCR Enzyme System (BD Biosciences Clontech, CA, USA). PCR amplicons were sequenced directly by Big Dye Terminator Version 3.1 (ABI, Tokyo, Japan) with universal M13 forward/M13 reverse primers using an ABI prism 3130 sequencer (ABI). The sequence files generated were assembled using Vector NTI software (Invitrogen) and base-calling errors were corrected following visual inspection of the chromatogram. When several peaks were observed at the same nucleotide position in the chromatogram, the highest chromatogram peak was read as the dominant nucleotide. In sequence analysis, multiple sequence alignment was performed with ClustalW, and the mean genetic distance was calculated using the p-distance algorithm in the MEGA version 4 DNA software. As a result, 60 genotype-2b HCV full open reading frame sequences were determined. In Table S1, obtained GenBank accession numbers for these sequences determined in this study are listed.

Table 4. Multivariate Logistic Regression Analysis.

Factor	odds (95% CI)	p value
Age	0.94 (0.85–1.04)	0.20
E2 aa 530 non T	4.33 (0.48–39.3)	0.19
NS5A aa 2359 N	3.22 (0.18–57.7)	0.43
NS5B 2631 non P	5.14 (0.29–91.2)	0.26
NS2 aa 879–893 mutations ≥2	9.77 (0.52–182)	0.13
NS5A aa 2045–2051 no mutations	4.46 (0.39–50.6)	0.23
NS5A aa 2224–2242 mutations ≥1	11.0 (1.13–107)	0.04
NS5A aa 2379–2405 mutations ≥1	7.03 (0.62–79.8)	0.12

To evaluate the optimal threshold of amino acid variations for SVR prediction in each viral region extracted, a receiver operating characteristic curve was constructed and the most optimal cut off value was determined for each region.
doi:10.1371/journal.pone.0024514.t004

Sliding Window Analysis

A sliding window analysis was introduced to search through HCV amino acid "regions", rather than single amino acid positions, related to the final outcome of PEG-IFN/RBV therapy. Briefly, the total number of amino acid substitutions compared to the consensus sequence within a given amino acid length were counted at each amino acid position in each HCV sequence. The consensus sequence was generated from these 60 patients. Then the relation of substitution numbers and the final outcome was compared statistically between the SVR and non-SVR groups by Mann-Whitney's U test for each amino acid position. In this study, we changed the window length from 1 to 50 to search for those HCV regions. To visualize the result, significantly lower p-values were colored in red and non-significant p-values were colored in green using Microsoft Excel software to generate a "heat map" appearance. In the present study, p-value of 1/300 or lower was colored in the maximum red.

Statistical Analysis

Statistical differences in the parameters, including all available patients' demographic, biochemical, hematological, and virological data such as sequence variation factors, were determined between the various groups by Mann-Whitney's U test for numerical variables and Fisher's exact probability test for categorical variables. To evaluate the optimal threshold of variations for SVR prediction, a receiver operating characteristic curve was constructed and the area under the curve as well as the sensitivity and specificity were calculated. Variables that achieved statistical significance ($p<0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. We also calculated the odds ratios and 95% confidence intervals. All p values of <0.05 by the two-tailed test were considered significant.

Results

Characteristics of the patients studied

The SVR rate of the patients analyzed was 75.9% (44/58) with the standard therapy (two non-SVR patients received extended therapy). The baseline characteristics of the patients classified according to achievement of SVR are shown in Table 1. Rapid virological response (RVR; undetectable serum HCV RNA within 4 weeks) and early virological response (EVR; undetectable serum HCV RNA within 12 weeks) rates were significantly higher in SVR patients ($p = 0.0008$ and 0.004). In addition, patients with non-SVR were older ($p = 0.04$). Pretreatment HCV RNA titer, which is known to affect the treatment outcome in genotype 1 and 2a HCV infection, did not differ significantly between two groups. Achievement of RVR reached 42.4% when all patients were included, and this rate was high compared to achievement of RVR in patients with genotype 1b infection (~10%) observed in

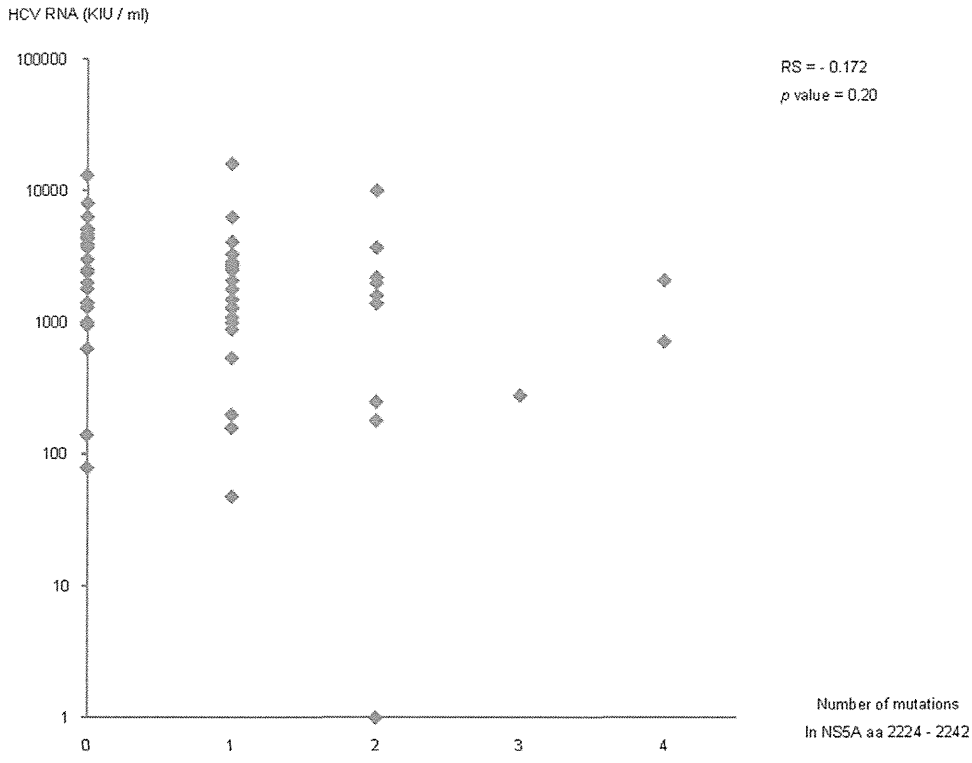

Figure 5. Correlation between pretreatment HCV RNA levels and the number of substitutions in the NS5A region aa 2224 to 2242. Spearman's correlation coefficient by rank test is demonstrated.
doi:10.1371/journal.pone.0024514.g005

University of Yamanashi (data not shown). The early virological response (EVR) rate was equally high in the SVR (97.7%) and non-SVR (68.8%) groups. Interestingly, most of the non-SVR patients (14/16, 87.5%) in genotype-2b HCV infection showed end-of-treatment response (ETR; undetectable serum HCV RNA at the end of therapy), demonstrating that the main cause of non-SVR was relapse (reappearance of hepatitis C viremia during the follow-up period after stopping therapy in patients with an ETR, n = 14), and not null response (detectable serum HCV RNA at the end of therapy, n = 2).

Phylogenetic analysis of SVR and non-SVR patients using the complete HCV amino acid sequence

To determine the viral sequence characteristics in the SVR and non-SVR groups, we first aligned all 60 HCV complete ORF amino acid sequences obtained from the patients' pretreatment sera along

Table 5. Baseline Characteristics of patients with NS5A aa 2224–2242 variations none or 1≤.

Characteristic	Variation 1≤ (n = 33)	No variation (n = 27)	*P* value
Gender (Male/Female)	17/16	18/9	NS[†]
Age (yrs)	57 (29–72)[*]	57 (22–80)	NS[‡]
ALT (IU/l)	72 (19–380)	47 (17–390)	NS[‡]
Platelet ($\times 10^4/mm^3$)	19.3 (7.1–31.8)	17.5 (10.4–36.7)	NS[‡]
Fibrosis score (0–2/≥3)[§]	26/5	19/3	NS[†]
HCV RNA (KIU/ml)	1600 (100–16000)	2450 (140–13000)	NS[‡]
IFN dose (≥80%/60–80%)	26/7	23/4	NS[†]
Ribavirin dose (≥80%/60–80%)	24/9	19/8	NS[†]
RVR rate (%)	53.1	29.6	NS[†]
EVR rate (%)	96.9	81.5	NS[†]
SVR rate (%)	90.9	51.9	0.001[†]
Replapse rate (%)	40.7	9.1	0.006[†]

[§]: 1≤ : n = 31, 0 : n = 22.
[*]: median (range).
[†]: Fisher's exact probability test.
[‡]: Mann-Whitney's U test.
doi:10.1371/journal.pone.0024514.t005